Carbon Dioxide-rich Water Bathing Increases Myonuclear Number and Muscle Fiber Size in Regenerating Skeletal Muscles

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Abstract. [Purpose] The purpose of this study was to investigate whether CO₂ water bathing accelerates skeletal muscle regeneration after injury. [Subjects] Wistar female rats (n=4/each group) were used in this study. [Methods] The rats were divided into non-injury (NI), injury (IC), injury + tap water bathing (ITW), and injury + CO₂ water bathing (ICO₂) groups. Skeletal muscle injury was induced by injection of bupivacaine hydrochloride in left tibial anterior (TA) muscles. Tap and CO₂ water (1,000 ppm) bathing was performed at 37 °C for 30 minutes once a day. Left TA muscles in all groups were removed at 2 weeks after injury, and then myonuclear number, myofiber size, and the expression of MyoD and myogenin were measured. [Results] Myonuclear number was increased in the ICO₂ group compared with the IC and ITW groups. Myofiber size in the IC, ITW, and ICO₂ groups was smaller than in the NI group, but it was larger in the ICO₂ group than in the IC group. The levels of expression of MyoD and myogenin were the same in all groups. [Conclusion] CO₂ water bathing may accelerate skeletal muscle repair after injury.

Key words: Carbon dioxide-rich water bathing, Muscle repair, Muscle injury

INTRODUCTION

Skeletal muscle has the ability to regenerate new muscle fibers after injury. Satellite cells are muscle stem cells and have an important role in skeletal muscle repair after injury. Satellite cells are located between the basal lamina and sarcolemma and are normally in a quiescent state. Satellite cells are activated when skeletal muscle fibers are injured and then divide, differentiate, and fuse to repair injured skeletal muscle fibers. Numerous factors have been implicated in the regulation of satellite cells during the proliferative and differentiation phases subsequent to muscle injury. The differentiation of satellite cells appears to be intimately tied to the myogenic basic helix-loop-helix family of transcription factors, in particular MyoD, myf5, myogenin, and MRF4. A number of studies have demonstrated a role for myf5 and MyoD in the determination of the satellite cell to a muscle lineage, while myogenin and MRF4 appear to be associated with the promotion of muscle differentiation¹).

Carbon dioxide-rich (CO₂) water bathing results in vasodilatation and an increase in blood flow², ³). The vasodilatation is induced by CO₂, which diffuses into the subcutaneous tissue through the skin layer. Gurevicius et al. reported that hypercapnic acidosis elicited release of nitric oxide (NO) from the coronary vascular endothelium⁴). This finding suggested that vasodilatation in response to CO₂ may be mediated in part by NO. Irie et al. reported that CO₂ water bathing increased phosphorylation of NO synthase (NOS) in ischemic skeletal muscle of mice⁵). This finding suggested that NO could be increased in skeletal muscle by CO₂ water bathing. NO mediates the rapid activation of Satellite cells in skeletal muscle to enter the cell cycle⁶). Such cycling provides new precursor cells for skeletal muscle growth and repair from injury⁷, ⁸). The role of NO in the release of HGF, which activates satellite cells, from the extracellular matrix has been investigated. NO is a key signal responsible for satellite cell activation and HGF transcription and release after skeletal muscle injury⁹). Taken together, CO₂ water bathing may accelerate skeletal muscle repair after muscle fiber injury.

We hypothesized that CO₂ water bathing could accelerate skeletal muscle repair after muscle fiber injury and activate satellite cells. Skeletal muscle fiber injury induces soreness, the loss of muscle contractility, and range of motion. Skeletal muscle repair occurs immediately after injury, but it is found to be very slow, and it takes a very long time to achieve complete recovery of skeletal muscle fiber. Therefore, the challenge to accelerate skeletal muscle repair after muscle fiber injury is meaningful and important. In the present study, we examined whether CO₂ water bathing accelerates skeletal muscle repair and increases the expression of MyoD and myogenin after injury.
SUBJECTS AND METHODS

Sixteen female Wistar rats (237–288 g) were used in this study. The rats were housed in a room with the temperature controlled at 22 ± 2 °C with a 12 h:12 h light-dark cycle. The rats had free access to food and water. This study was approved by the Animal Use Committee of Kibiti International University.

The rats were assigned to non-injury (NI, n=4), injury (IC, n=4), injury + tap water bathing (ITW, n=4), and injury + CO₂ water bathing (ICO₂, n=4) groups. The rats in the IC, ITW, and ICO₂ groups were anesthetized by injection of pentobarbital sodium (50 mg/kg) and then the left tibial anterior (TA) muscle was injured by injection of 0.3 mL of 0.5% bupivacaine hydrochloride (Marcaine, AstraZeneca, Osaka, Japan) using a disposable syringe with a 27-gauge needle. The needle was inserted into the mid-belly portion of the TA muscle and advanced longitudinally to the proximal portion. The solution was injected as the needle was withdrawn slowly as described previously[10]. In the present study, we used bupivacaine hydrochloride to induce similar degrees of muscle injury. In a preliminary experiment, we confirmed that the TA muscle injuries in rats were of similar degree at 4 day after the injection of bupivacaine hydrochloride using microscopy.

The rats in the ITW and ICO₂ groups were immersed in tap water and CO₂ water at 37 °C for 30 minutes, respectively. CO₂ water containing a high concentration of CO₂ (1,000 ppm) was made from high pressure CO₂ in a cylinder and tap water by using an MRE-Spa (Mitsubishi Rayon Co., Ltd., Tokyo, Japan). Both water batings were started at one day after injection and performed once a day, five times a week at −20 °C. The sections were air-dried and fixed with 10% formaldehyde in 0.1 M PBS (pH 7.4) for 15 minutes. They were then stored at −80 °C until measurement.

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Serial cross sections (10 μm thick) were cut in a cryostat at −20 °C. The sections were air-dried and fixed with 10% formaldehyde in 0.1 M PBS (pH 7.4) for 15 minutes. They were then washed in 0.1 M PBS for 10 minutes and incubated with 10% normal serum and 1% Triton X-100 for 1 hour at room temperature to block nonspecific staining. Next, they were incubated with primary mouse monoclonal anti-dystrophin antibody (Sigma, St. Louis, MO, USA) diluted 1:100 with 0.1 M PBS for 5% normal serum and 0.3% Triton X-100 for 2 hours at room temperature to identify the plasma membrane. The sections were then washed 3 times in 0.1 M PBS and incubated with the secondary Alexa Fluor 488 anti-mouse IgG antibody (CTS Japan, Tokyo, Japan) diluted 1:100 with 0.1 M PBS with 5% normal serum and 0.1% Triton X-100 for 60 minutes at room temperature. The sections were washed 3 times in 0.1 M PBS and then mounted with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The number of DAPI-labeled myonuclei located within the plasma membrane was determined from at least 100 fibers and expressed as the myonuclear number per fiber cross section as described previously[12]. The cross-sectional area of muscle fibers was measured using the ImageJ software (NIH, Bethesda, MD, USA) for at least 100 fibers and expressed as the mean fiber cross-sectional area.

For analysis of the expression of MyoD and Myogenin, the muscles were homogenized in Tris-HCl (pH 7.4). After centrifugation, the supernatant was collected as the measurement sample. Protein content in these samples was measured by the Bradford method using a Coomassie Protein Assay kit (Thermo Fisher Scientific K.K., Kanagawa, Japan). The samples were added to EzApply (ATTO, Tokyo, Japan), adjusted to a final protein concentration of 1 μg/μl, and then boiled at 95 °C for 5 minutes. We then applied 10 μg protein to a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan). Electrophoresis was carried out at a constant current of 20 mA/gel for 90 minutes, and proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a HorizBLOT 2 M (ATTO, Tokyo, Japan) at a constant current of 2 mA/cm² for 60 minutes. Following blots, the PVDF was then incubated for 60 minutes using EzBlot (ATTO, Tokyo, Japan). The membrane was then incubated with a monoclonal antibody for MyoD (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2,000 with 0.1 M Tris-HCl (pH 7.5) or myogenin (Santa Cruz Biotechnology, Sanata Cruz, CA, USA) diluted 1:2,000 with 0.1 M Tris-HCl (pH 7.5) for 1 hour at room temperature. The membrane was washed 3 times in 0.1 M Tris-HCl with 0.1% Tween-20 and then reacted with anti-mouse IgG (Nacalai Tesque, Kyoto, Japan) diluted 1:10,000 with 0.1 M Tris-HCl (pH 7.5) for 1 hour at room temperature. The membrane was washed 3 times in 0.1 M Tris-HCl with 0.1% Tween-20 and then reacted with EzWestBlue (ATTO, Tokyo, Japan). Bands from Western blots were quantified using the ImageJ software.

Data were expressed means ± standard deviation and were analyzed by one-way ANOVA. When there were significant differences, the Tukey post hoc test was used to determine differences between the groups. Statistical analyses were performed using Excel Statistics 2008 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical significance was set at p<0.05.

RESULTS

All data are shown in Table 1. The mean fiber cross-sectional area was significantly smaller in the IC (55%), ITW (51%), and ICO₂ (38%) groups than in the NI group. In the ICO₂ group, the mean fiber cross-sectional area was significantly larger (37%) than in the IC group. The mean fiber cross-sectional area was higher in the ICO₂ group than in the ITW group, but a significant difference was not observed between these groups (p =0.13). The mean myonuclear...
number per fiber cross section was significantly lower in the IC (25%) and ITW (26%) groups than in the ICO2 group. The mean myonuclear number per fiber cross section was significantly higher than in the NI (33%) and ITW (35%) groups. There were no significant differences in the protein concentrations of MyoD and myogenin between any groups.

**DISCUSSION**

CO2 water bathing accelerated the recoveries of skeletal myonuclear numbers and skeletal muscle cross-sectional area at 2 weeks after skeletal muscle injury. These findings indicate that CO2 water bathing may accelerate recovery from skeletal muscle injury.

NO is a key signal responsible for satellite cell activation and HGF, which is an activator of satellite cells, release from the extracellular matrix after skeletal muscle injury9). Tatsumi et al. reported that activation of satellite cells and HGF in stretched muscle were dependent on local NO production13). Irie et al. reported that CO2 water bathing increased the phosphorylation of NOS in ischemic skeletal muscle5), indicating that CO2 water bathing promotes NO production in skeletal muscle. Although no measure of NOS was included in the present study, we speculated that NO production in skeletal muscle was promoted by CO2 water bathing. Satellite cell activation is involved in increasing myonuclear number14). In the present study, the myonuclear number per fiber cross section was decreased in the IC and ITW groups at 2 weeks after injury compared with in the NI group. The value in the ICO2 group recovered to the same level as in the ITW groups at 2 weeks after injury compared with in the NI group.

In conclusion, CO2 water bathing after muscle injury may accelerate muscle fiber repair. Skeletal muscle fiber repair after injury seems to be very slow. Skeletal muscle injury occurs in rehabilitation of disuse muscle atrophy, sports, etc. Muscle injury reduces force generation and range of motion, and these impairments can lead to activity limitations and performance decreases in athletes. Therefore, acceleration of skeletal muscle repair is very important. CO2 water bathing may be a useful hydrotherapy for patients with muscle injury to accelerate repair.

As a limitation of our study, we measured several data at 2 weeks after muscle injury. At 2 weeks after injury, muscle fiber repair had not yet completed. We expected that CO2 water bathing would accelerate skeletal muscle repair, and many myofibers without central nuclei, which indicates complete repair of injured myofibers, could be observed compared with nontreatment at 4 weeks after injury. The levels of expression of MyoD and myogenin at 2 weeks after injury were similar in non-injured muscle. Taken together, further study is needed at several time points to determine the effect of CO2 water bathing on skeletal muscle repair after injury.

**REFERENCES**


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**Table 1.** Mean fiber cross-sectional area, myonuclear number per fiber cross section, and expression of MyoD and myogenin

<table>
<thead>
<tr>
<th></th>
<th>Fiber cross-sectional area (μm²)</th>
<th>Myonuclear / fiber cross section</th>
<th>MyoD (% of control)</th>
<th>Myogenin (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NI (n=4)</td>
<td>2112 ± 202</td>
<td>1.68 ± 0.10</td>
<td>100 ± 18</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>IC (n=4)</td>
<td>961 ± 75*</td>
<td>1.27 ± 0.13*</td>
<td>120 ± 21</td>
<td>99 ± 18</td>
</tr>
<tr>
<td>ITW (n=4)</td>
<td>1049 ± 143*</td>
<td>1.26 ± 0.13*</td>
<td>113 ± 31</td>
<td>97 ± 16</td>
</tr>
<tr>
<td>ICO2 (n=4)</td>
<td>1317 ± 190* #</td>
<td>1.79 ± 0.11 #</td>
<td>124 ± 22</td>
<td>104 ± 16</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation. *Different from the NI group at p<0.01. †Different from the IC group at p<0.05 or 0.01, respectively. ‡Different from the ITW group at p<0.01.